PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Jean J. Frechet, *et al.*

Application No.: 09/963,858

Filed: September 25, 2001

For: DENDRIMERIC SUPPORT OR CARRIER MACROMOLECULE

Customer No.: 43850

Confirmation No. 1612

Examiner: Riley, Jezia

Technology Center/Art Unit: 1637

DECLARATION OF PROFESSOR FRECHET UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Jean Frechet, Ph.D. declare as follows:

1. I am a Professor of Chemistry at the University of California at Berkeley. I have published over 200 scientific articles related to dendrimers and other dendritic polymers. My *Curriculum Vitae* is attached.

2. I am an inventor of the subject matter claimed in the U.S. Patent Application No. 09/963,858.

3. I have reviewed the Office Actions that were mailed on February 23, 2005 as well as September 6, 2005, as well as the references cited in those Office Actions. I understand the content of these references.

4. The only cited reference which describes the particular dendrimer subunit of my invention is Annby, *et al.*, *Tetrahedron Letters*, **39**: 3217-3220 (1998) ("Annby"). The other references cited in previous Office Actions either do not describe this dendrimer subunit

(Tomalia) or describe hyperbranched polymers rather than dendrimers (Magnusson, Hult and Trollsas). Since Annby is the closest art located in the search, Annby, and its production of a dendrimer composition that is not free of urea side products, will be the focus of this discussion.

5. It is my understanding that the dendrimers of Annby are produced via a dendrimer generation step involving a carbodiimide (DCC). Carbodiimides react with the dendrimers in the generation step to form a mixture of complete dendrimers, dicyclohexylurea, and N-acyl urea dendrimer side products. The invention claimed in U.S. Patent App. No. 09/963,858 is directed to dendrimers in which carbodiimides are not used in the dendrimer generation step. Thus the mixture is free of urea dendrimer side products. Therefore, I am submitting this declaration in order to illustrate the issues with these side products.

6. The side product issues involved with carbodiimides such as DCC are well known in the art. I mentioned this problem in the Background section of U.S. Pat. App. No. 09/963,858:

> Ester formation via carbodiimide coupling of the monomer to the initiator molecule produces N-acyl urea-containing side products as well as the desired dendrimer. In addition the reaction with dicyclohexyl carbodiimide produces a very large amount of dicyclohexyl urea which is very difficult to remove from the desired dendrimer product. The formation of the side products is particularly problematic for the multistep synthesis of a polymeric species such as a dendrimer; the N-acyl urea side product formed in a first step reacts in a subsequent step adding a new generation to both the desired dendrimer core and to the side product. The similarity in properties between the side product and the desired dendrimer makes separation of the species difficult, and economically unfeasible for the large-scale preparation of dendrimers. As dendrimers are under intense evaluation as delivery vehicles for therapeutic and diagnostic agents, the presence of the N-acyl urea side products as well as any dicyclohexylurea impurity is seen as a serious impediment to understanding the pharmacokinetics and pharmacodynamics of these important agents, and will surely hamper their progress towards regulatory approval.
> U.S. Patent App. No. 09/963,858, Page 2 lines 4-17

7. The side product issues are also described by others in the art, such as Radau, *Monatshefte fur Chemie,* **134**, 1033-1036 (2003) ("Radau"). Radau discusses the purification problems which plague DCC coupling reactions:

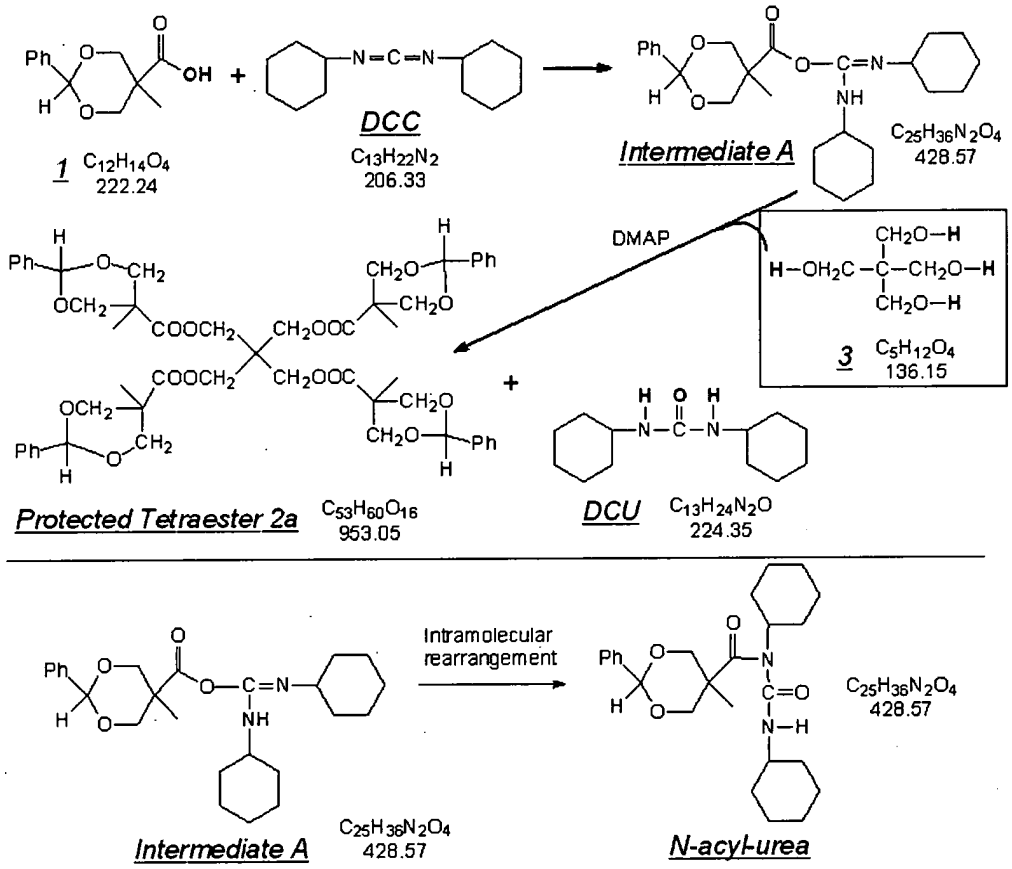
> DCC is the most frequently used coupling reagent of the carbodiimide type. In contradiction to this impressive fact, there are several shortcomings to the DCC method. The N,N'-dicyclohexylurea by product, while indeed insoluble in most organic solvents (except alcohols) and thus removable by filtration, is not entirely insoluble and ***therefore it frequently contaminates the product. A more disturbing side reaction*** is the intramolecular CO-N shift in the O-acyl isourea intermediate ***yielding an N-acylurea derivative as a by-product.***
>
> Radau, p. 1033, (emphasis added)

Radau was cited in our Office Action Response dated February 7, 2006, and it is included with this Declaration.

8. As mentioned, Annby uses DCC in its dendrimer-generation step. Annby teaches the preparation of "1G (generation) dendrimer 2" by acylation of 1 equivalent of pentaerythritol with 8 equivalents of acetal ***1***, 0.5 equivalent of DMAP, and 7.5 equivalents of *DCC* (dicyclohexylcarbodiimide) in methylene chloride at room temperature for four days.

9. This reaction shown in Scheme 1 proceeds by reaction of ***1*** with DCC to form an ***intermediate A***.

Scheme 1



Since 7.5 equivalents of ***DCC*** are used, only 7.5 equivalents of ***intermediate A*** can be formed and 0.5 equivalent of ***1*** remains in the reaction mixture. ***Intermediate A*** then reacts with 1 equivalent of pentaerythritol ***3***. Since pentaerythritol contains four OH groups, four equivalents of ***intermediate A*** are consumed and 3.5 equivalents remain. Therefore the product of this reaction consists of 1 equivalent of ***protected tetraester 2a***, 4 equivalents of dicylclohexylurea (***DCU***) side-product, 3.5 equivalents of ***intermediate A***, 0.5 equivalent of DMAP, and 0.5 equivalent of ***1***.

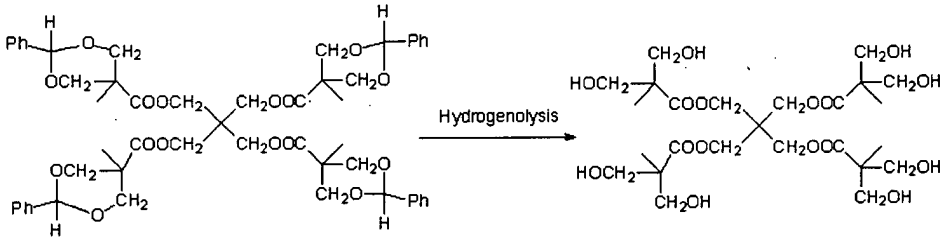
10. Annby then teaches removal of ***DCU*** by filtration followed by washing with $NaHCO_3$ and crystallization to afford the ***protected tetraester 2a***. It is well known in the art that such filtration cannot remove all of the ***DCU*** since some always remains dissolved. In addition a

large amount of ***intermediate A*** and compound ***1***, which can react again to produce more ***DCU***, remain in the reaction mixture.

11. It is also well-known in the art (see M. Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, Berlin, 1984, p. 37-38) that ***intermediate A*** can undergo an intramolecular rearrangement to yield an ***N-acyl-urea*** derivative as a by-product as shown in Scheme 1. Since a large amount of ***intermediate A*** is always present because Annby teaches the use of a large excess of both ***1*** and ***DCC*** with respect to pentaerythritol ***3***, this ***N-acyl-urea*** is a contaminant found in the product. ***N-acyl-urea*** may also be produced by reaction of ***DCU*** with ***intermediate A***, both of which are present in large amount in the reaction product of Annby.

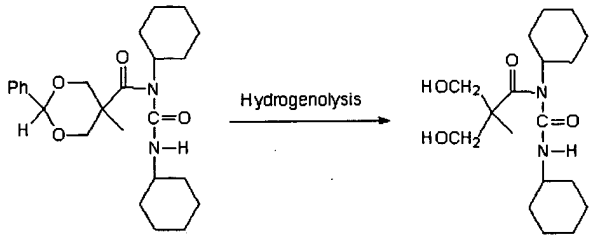
12. Scheme 2 shows the final step in the preparation of "***1G*** (generation) ***dendrimer 2***" by hydrogenolysis of the benzylidene protecting groups of ***protected tetraester 2a***. Note that in this reaction the protecting group of the ***N-acyl-urea*** by-product is also removed affording a contaminant molecule that can react in the subsequent generation growth step of the dendrimer.

Scheme 2.



Protected Tetraester 2a $C_{53}H_{60}O_{16}$ 953.05

1G dendrimer 2 $C_{25}H_{44}O_{16}$ 600.61



N-acyl-urea $C_{25}H_{36}N_2O_4$ 428.57

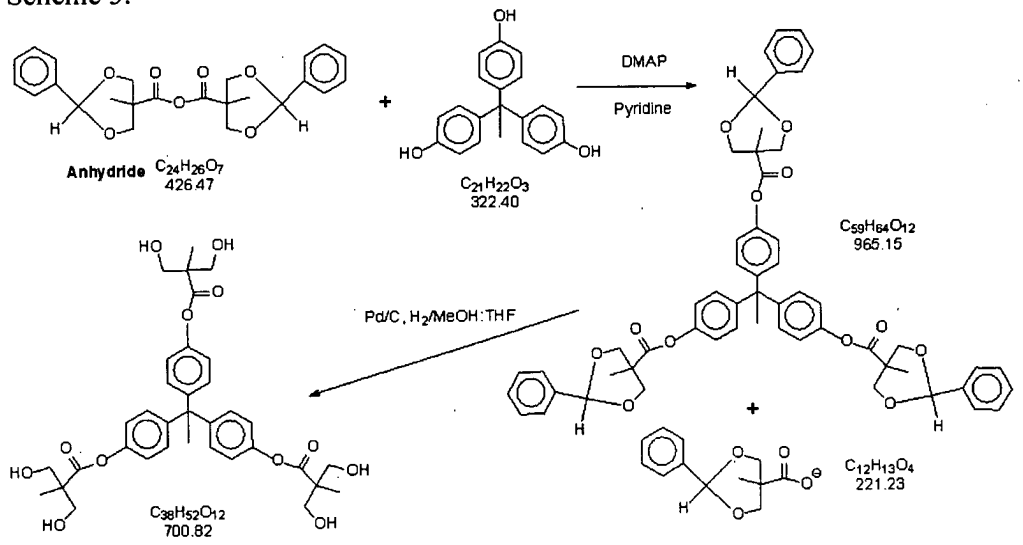
1G side product $C_{18}H_{32}N_2O_4$ 340.46

13. The amounts of undesired products obtained in the Annby preparation can be calculated using the molar masses of the individual compounds and the respective amounts taught by Annby. Thus, to produce 100 g of dendrimer ***2***, Annby would teach using 296 g of ***1***, and 258 g of ***DCC*** with 22.7 g of pentaerythritol 3, After 4 days of reaction as taught by Annby at least 149g of ***DCU*** would be contained in the product as well as 18.5 g of **1**, and 250 g of ***intermediate A*** or the ***N-acyl-urea*** compound which is formed spontaneously from ***intermediate A***. Thus a total of 399 g of coupling agent derived side products are found in the reaction mixture used to produce 100g of ***dendrimer 2***.

14. In contrast our procedure involves a pure anhydride which is free of DCU or any other contaminant CANNOT produce a material contaminated by DCU or an N-acyl-urea side product since no DCC is used to prepare the dendrimer. Therefore no coupling agent derived side product is found since no coupling agent is used in this procedure.

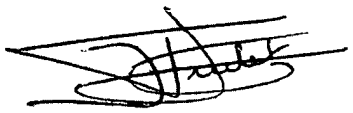
15. As shown in Scheme 3, representing example 2 of our specification, the anhydride reacts directly with the compound 1,1,1-Tris(hydroxyphenyl)ethane containing three OH groups and the desired product is obtained in 97% yield while no impurities can be detected by mass spectrometry, nuclear magnetic resonance spectroscopy, infrared spectroscopy or elemental analysis as described in the specification.

Scheme 3.



16. In contrast to Annby, my invention employs an anhydride of the dendrimer subunit in the dendrimer generation step. The use of this anhydride obviates the need for carbodiimides in the dendrimer generation step, which is what allows my invention to be substantially free of the urea side products which plagued earlier dendrimer syntheses described in the art and exemplified by Annby.

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Dated: February 28, 2006

Jean Frechet, Ph.D.

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Suppression of Formation of *N,N'*-Dicyclohexylurea Derivatives During *DCC*-Activation of Proline-Containing Dipeptides

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Summary. The syntheses of dipeptide esters containing a *C*-terminal *L*-proline moiety using carbodiimides as coupling reagents strongly depend on the choice of appropriate conditions. Thus, the use of *DCC* prefers the formation of the undesirable *N,N'*-dicyclohexylurea derivative **3** as a consequence of a CO → N-shift in the *O*-acyl isourea intermediate instead of the desired dipeptide ester **4**. In our hands, only *DIC* was able to yield the desired product exclusively.

Keywords. Carbodiimide; Dicyclohexylurea; Ester synthesis.

Introduction

Since the first report on the utilization of *N,N'*-dicyclohexylcarbodiimide (*DCC*) as a reagent that can effect the formation of peptide bonds in 1955 [1], *DCC* is the most frequently used coupling reagent of the carbodiimide type. In contradiction to this impressive fact, there are several shortcomings of the *DCC* method. The *N,N'*-dicyclohexylurea by-product, while indeed insoluble in most organic solvents (except alcohols) and thus removable by filtration, is not entirely insoluble and therefore it frequently contaminates the product. A more disturbing side reaction is the intramolecular CO → N-shift in the *O*-acyl isourea intermediate yielding an *N*-acylurea derivative as by-product.

Herein, we report on the formation of *N,N'*-dicyclohexylurea derivatives as by-products during the activation of proline-containing dipeptides and on successfull variations of the reaction conditions which result (1) in an increased selectivity in

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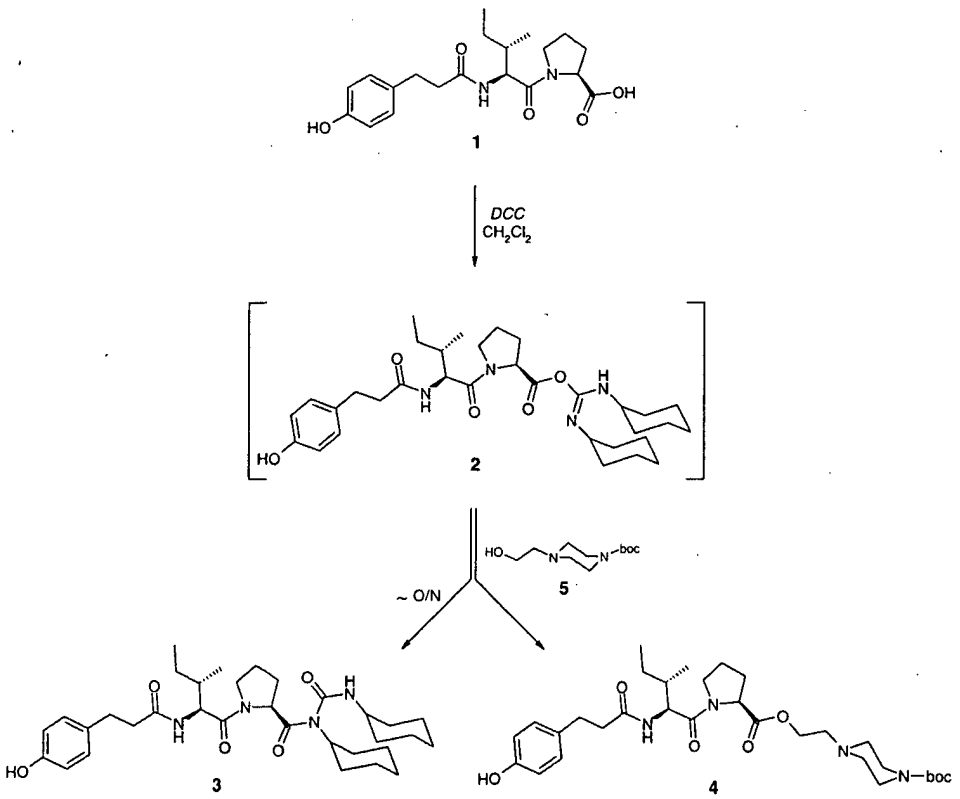
favour of the desired product, and (2) in a complete suppression of the formation of undesirable urea derivatives.

Results and Discussion

In the course of our continuing studies towards the synthesis of cyanopeptide-derived inhibitors of trypsin-like serine proteases [2–4], **1** was synthesised as a common intermediate scaffold of diverse target molecules [5]. Thus, **1** was activated *in situ* by means of *DCC* to the corresponding *O*-acylisourea **2** in order to react with nucleophiles like alcohols or amines (*e.g.* **5**, Scheme 1) to esters **4** or amides.

DCC turned out to be not suitable for catalyzing the coupling reaction effectively, but this statement depends on the reaction conditions. Using the 1-hydroxybenzotriazole (HO*Bt*)/*DMF* protocol, *DCC* exclusively allows the formation of **3** (Table 1) whose proline moiety is not provided with sufficient carbonyl reactivity to react with the alcohol – in contrast to **2**. When compared to the esterification with **5** to give **4**, the CO → N-rearrangement to **3** is the exclusive reaction that takes place. Compared to the desired product **4**, urea **3** possesses excellent crystallization properties.

The use of *DCC*/dichloromethane results in a little satisfying mixture of **3** and **4**. Separation and purification of this mixture by column chromatography requires a high degree of experience and talent.



Scheme 1

Table 1. Influence of reaction conditions on product formation

Conditions	3 %*	4 %*
DCC/CH_2Cl_2	19	29
DIC/CH_2Cl_2	0	55
DCC/HO*Bt*/*DMF*	35	0

* Yield related to **1**

The combination of *N,N'*-diisopropylcarbodiimide (*DIC*)/dichloromethane in our hands was the most successful variation. The desired product **4** was the only product formed with a yield of 55% and purification by chromatography worked without any problems. The formation of an analogous *N,N'*-diisopropylurea derivative was not detectable.

In conclusion, we have shown that the formation of esters of peptide derivatives containing a *C*-terminal *L*-proline moiety using carbodiimides as coupling reagents strongly depends on the choice of appropriate reaction conditions. Predictions concerning the course of the reaction and the suppression of formation of by-products still maintain difficult.

Experimental

General: Melting points are not corrected. IR spectra (KBr): IR spectrometer Perkin-Elmer 1600 series FTIR. NMR spectra: Bruker DPX 300 (300 MHz), solvent: $CDCl_3$, internal standard: *TMS*. Elemental analyses: Perkin-Elmer Elemental Analyzer 2400 CHN, all compounds gave satisfactory elemental analyses. Chromatography: cc: Merck silica gel 60 (0.063–0.200 mm). Optical rotation ($[\alpha]_D$): Polartronic D (Schmidt Haensch GmbH).

Abbreviations of amino acids follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [6]. Other abbreviations: *Boc: tert.*-Butyloxycarbonyl, *DCC*: *N,N'*-dicyclohexylcarbodiimide, *DIC*: *N,N'*-diisopropylcarbodiimide, *EtOAc*: ethyl acetate, *PE*: petroleum ether.

1-[N-[3-(4-Hydroxyphenyl)propionyl]-L-isoleucyl-L-prolyl]-1,3-dicyclohexylurea (**3**, $C_{33}H_{50}N_4O_5$)

Colourless crystals, mp: 79–81°C; yield: 19% (*DCC*/CH_2Cl_2), 35% (*DCC*/HO*Bt*/CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.79$ (d, $J = 7.2$ Hz, *Ile*-CH-C$\underline{H}_3$), 0.94 (t, $J = 7.4$ Hz, *Ile*-CH_2-C$\underline{H}_3$), 1.00–2.30 (m, *Pro*-β-C$\underline{H}_2$-γ-C$\underline{H}_2$, *Ile*-β-H, *Ile*-C$\underline{H}_2$-CH_3, 2x C_6H_{11}), 2.32–2.45 (m, *Ph*-CH_2-C$\underline{H}_2$), 2.80–2.87 (m, *Ph*-C$\underline{H}_2$-CH_2), 3.60–3.70 (m, *Pro*-N-C$\underline{H}_2$), 3.80–3.90 (m, *Pro*-N-C$\underline{H}_2$), 4.54–4.64 (m, *Pro*-α-H), 4.78–4.83 (m, *Ile*-α-H), 6.07 (d, NH), 6.73 (d, $J = 8.4$ Hz, $H_{arom.}$), 6.98 (d, $J = 8.4$ Hz, $H_{arom.}$), 7.71 (d, $J = 7.3$ Hz, NH_{urea}) ppm; IR (KBr): 3323, 3040, 2931, 2854, 1708, 1657, 1626, 1516, 1450, 1381, 1342, 1296, 1258, 1227, 1162, 1081, 1050, 987, 893, 831, 642, 534 cm^{-1}; $[\alpha]_D^{23} = +25.66$ ($c = 2$, *Me*OH).

N-[3-(4-Hydroxyphenyl)propionyl]-L-isoleucyl-L-proline 2-[4-(tert.-butyloxycarbonyl)-piperazin-1-yl]ethyl ester (**4**, $C_{31}H_{48}N_4O_7$)

Typical procedure (*DCC*/CH_2Cl_2 and *DCC*/HO*Bt*/CH_2Cl_2 methods were carried out in an analogous way): A solution of 0.56 g of *DIC* (4.40 mmol) in 30 cm^3 of CH_2Cl_2 was added dropwise to a stirred,

ice-cooled mixture of 1.65 g of **1** (4.40 mmol) in 120 cm^3 of CH_2Cl_2 over a period of 20 min and was stirred for one additional hour. Compound **5** (1.11 g, 4.84 mmol) in 70 cm^3 of CH_2Cl_2 was added dropwise over a period of 2 h. While stirring over night, the mixture was allowed to warm up to room temperature. The solvent was evaporated under reduced pressure and the resulting residue was purified by chromatography (silica gel, eluent: CH_2Cl_2:*EtOAc*:*PE*:*Me*OH 10:10:10:1). Yield: 1.42 g (55%) of a colourless substance, mp: 60°C; ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 0.84$ (t, $J = 7.3$ Hz, *Ile*-CH-C$\underline{H}_3$), 0.95 (d, $J = 6.8$ Hz, *Ile*-CH_2-C$\underline{H}_3$), 1.00–1.15 (m, *Ile*-C$\underline{H}_2$-CH_3), 1.30–1.45 (m, *Ile*-C$\underline{H}_2$-CH_3), 1.46 (s, *t*-butyl), 1.60–1.75 (m, *Ile*-β-H), 1.85–2.25 (m, *Pro*-β-CH_2-γ-CH_2), 2.30–2.50 (m, $OCH_2C\underline{H}_2N$ + $N(C\underline{H}_2CH_2)_2N$-*Boc*), 2.58–2.64 (m, *Ph*-CH_2-C$\underline{H}_2$), 2.79–2.85 (m, *Ph*-C$\underline{H}_2$-CH_2), 3.36–3.44 (m, $N(CH_2C\underline{H}_2)_2N$-*Boc*), 3.50–3.60 (m, *Pro*-N-C$\underline{H}_2$), 3.80–3.90 (m, *Pro*-N-C$\underline{H}_2$), 4.24 (t, $J = 5.9$ Hz, $OC\underline{H}_2$), 4.41 (dd, $J = 8.3$, 3.5 Hz, *Pro*-α-H), 4.82 (dd, $J = 9.0$, 5.2 Hz, *Ile*-α-H), 6.14 (d, $J = 9.1$ Hz, NH), 6.73 (d, $J = 8.6$ Hz, $H_{arom.}$), 6.98 (d, $J = 8.6$ Hz, $H_{arom.}$) ppm; IR (KBr): 3293, 2970, 2934, 2876, 1748, 1698, 1628, 1516, 1453, 1366, 1245, 1171, 1130, 1004, 864, 830, 770 534 cm^{-1}; $[\alpha]_D^{23} = -32.83$ ($c = 2$, *Me*OH).

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